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Construction of a Baculovirus Derivative to Produce Linearized *Antheraea pernyi* (Lepidoptera: Saturniidae) Multicapsid Nucleopolyhedrovirus Genomic DNA

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Abstract

In the *Antheraea pernyi* multicapsid nucleopolyhedrovirus (AnpeNPV)-based expression vector system, the frequency of homologous recombination events between wild-type AnpeNPV DNA and the transfer vector is low, resulting in a small amount of recombinant virus. Previous reports have indicated that linearized baculovirus DNA can increase the proportion of recombinant virus relative to the total progeny. To improve the recombination efficiency, we constructed a linearized derivative of AnpeNPV, referred to as AnpeNPV^{PhEGFP}-*Avr*II, in which *egfp* flanked by *Avr*II restriction sites was located at the polyhedrin locus and driven by the polyhedrin promoter. Linear AnpeNPV DNA was obtained by the treatment of AnpeNPV^{PhEGFP}-*Avr*II genomic DNA with *Avr*II endonuclease. The infectivity and recombinogenic activity between the linearized and circular viral DNA were evaluated by quantitative real-time polymerase chain reactions. We demonstrated that the linearized AnpeNPV DNA produced only small numbers of infectious budded viruses, accounting for approximately 4.5% of the budded virus production of wild-type AnpeNPV DNA in *A. pernyi* pupae. However, the linearized AnpeNPV DNA substantially increased recombinant virus production after cotransfection with an appropriate transfer vector; relative abundance of the recombinant virus was approximately 5.5-fold higher than that of the wild-type AnpeNPV DNA in *A. pernyi* pupae. The linearization of AnpeNPV DNA will facilitate the purification of recombinant viruses using the AnpeNPV-based expression vector system and the construction of an AnpeNPV-based bacmid system.

Key words: *Antheraea pernyi*, nucleopolyhedrovirus, linearization, homologous recombination, quantitative real-time polymerase chain reaction

Nucleopolyhedroviruses are a type of baculovirus with a large, circular, double-stranded DNA genome of approximately 80–180 kb containing more than 100 genes. They infect just one or a few closely related insect species, mainly of the order Lepidoptera (Rohrmann 2019). The virus is widely used as a biopesticide in agriculture and as a protein expression vector in the baculovirus expression vector system (BEVS) for recombinant protein production with broad research and commercial applications. Its advantages as a protein expression vector include high levels of protein expression, cloning of large DNA fragments, and safety (Lemaitre et al. 2019). *Autographa californica* multiple nucleopolyhedrovirus (AcMNPV) was first used as a protein expression vector to produce human β-interferon in *Spodoptera frugiperda* 21 (Sf21) cells in 1983 (Smith et al. 1983). Shortly thereafter, *Bombyx mori* NPV (BmNPV) was used to produce human α-interferon in silkworm larvae (Maeda et al. 1985). BEVS has since become a powerful platform for recombinant protein production.

The nucleopolyhedrovirus has been continuously optimized to improve the generation and selection of recombinant viruses and to increase the yield and quality of recombinant proteins. For example, the recombinant virus generated in Sf21 cells by homologous recombination between the wild-type viral DNA and a transfer vector accounted for less than 0.1% of the total viruses in an early study (Smith et al. 1983). The recombinant virus was isolated by several rounds of plaque selection. To increase the percentage of the recombinant virus, a linearized derivative of AcMNPV has been obtained by introducing a Bsu36I restriction site into the polyhedrin locus of AcMNPV. Up to 30% of progeny viruses were recombinants when the linearized AcMNPV DNA was used for co-transfection with the transfer vector (Kitts et al. 1990). The rate of recombinant virus could be further increased to 80% or higher using linearized AcMNPV DNA with interruption of an essential gene (*orf1629*) for co-transfection (Kitts and Possee 1993). An important breakthrough for the recombinant baculovirus construction was the

development of the Bac-to-Bac system based on site-specific transposition technology (Luckow et al. 1993). It can generate 100% pure recombinant bacmid containing the AcMNPV genome in *Escherichia coli* and requires no downstream plaque purification for recombinant viruses in insect cells. Another major advance in the production of recombinant baculovirus was the development of the *flash*BAC system, which combines bacmid technology with homologous recombination (Possee et al. 2008). The major advantages of this system over Bac-to-Bac are that it eliminates the need for the transposase-encoding helper plasmid and recombinant bacmid screening in *E. coli*.

Antheraea pernyi multicapsid nucleopolyhedrovirus (AnpeNPV) is a baculovirus that specifically infects *A. pernyi*, commonly known as the Chinese oak silkworm, an economically important insect for silk production in China. *Antheraea pernyi* exhibits pupal diapause. The large size, year-round availability, and ease of manipulation, without the need for rearing, make them practical bioreactors. *Antheraea pernyi* is a permissive host of AnpeNPV and is not susceptible to AcMNPV or BmNPV (Liaoning Institute of Sericulture 2003). The AnpeNPV genome is 126,246 bp and encodes 145 predicted open reading frames (ORFs) (Fan et al. 2007); it has been developed as a baculovirus expression vector for recombinant protein production in *A. pernyi* pupae (Wang et al. 2010, Ye et al. 2014). AnpeNPV can infect nonpermissive High Five (BTI-TN-5B1-4, Tn-Hi5) host cells derived from *Trichoplusia ni* with reduced infectivity during serial passages but not cells from *Bombyx mori* or *Spodoptera frugiperda* (e.g., Sf9 and Sf21) (Zhao et al. 2019). Tn-Hi5 cells are now used to produce and select recombinant viruses in the AnpeNPV-based expression vector system. Recombinant viruses are generated in Tn-Hi5 cells by homologous recombination between AnpeNPV DNA harboring the enhanced green fluorescent protein (EGFP) gene driven by the polyhedrin promoter at the polyhedrin locus and a polyhedron-based transfer vector; these need to be propagated in *A. pernyi* pupae. The recombinants mixed with parental viruses from the infected pupae are further diluted by an end-point dilution method to infect Tn-Hi5 cells, and cells lacking EGFP expression are selected for isolation of the recombinant virus. The low efficiency and time-consuming purification of Tn-Hi5 cells from pupae, requiring several downstream steps, have seriously hampered research and the practical utilization of the AnpeNPV-based expression vector system. Therefore, it is necessary to improve the recombinant virus rate in the AnpeNPV-based expression vector system.

In this study, we engineered a derivative of AnpeNPV in which *egfp* flanked by *Avr*II restriction endonuclease sites was inserted at the polyhedrin locus. The infectivity of the linearized AnpeNPV DNA and the ability to generate recombinant viruses with transfer vectors were investigated. In comparison with wild-type AnpeNPV DNA, the linearized AnpeNPV DNA produced a higher proportion of recombinant AnpeNPV after co-transfection with an appropriate transfer vector in *A. pernyi* pupae.

Materials and Methods

Virus, Viral DNA, and Cell Lines

AnpeNPV L2 (GenBank accession number: EF207986) was propagated in *A. pernyi* pupae and viral DNA was extracted as previously described (Fan et al. 2007). Tn-Hi5 cells (BTI-TN-5B1-4) purchased from Thermo Fisher Scientific (Waltham, MA) were cultured in TNM-FH medium (GE Healthcare Life Sciences, Chicago, IL) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco, Billings,

MT) containing 0.5% penicillin–streptomycin solution (Gibco) at 27°C. Restriction enzymes were purchased from TaKaRa (Dalian, China).

Construction of Baculovirus Transfer Vectors

The pUC19 vector was used as a backbone to construct a baculovirus transfer vector. Two DNA fragments, one containing partial *orf144* (*lef-2*), full-length *orf145*, and the polyhedrin promoter (corresponding to nt 125168–126244) and another containing partial *orf1* (*polyhedrin*) and *orf2* (*orf1629*) (corresponding to nt 250–1268), were amplified from AnpeNPV DNA with the primer pairs Pap*Hind*III-F/Pap*Bam*HI- *Avr*II-R and Pap*Kpn*I-*Avr*II-F/ Pap*Eco*RI-R, respectively (Table 1), and cloned into pUC19 to create the vector pApPh/*Avr*II. The *egfp* gene was amplified from the viral DNA of *ApNPV-Δph/egfp⁺* (Zhao et al. 2019) by PCR with Pegfp-*Bam*HI and Pegfp-*Kpn*I primers (Table 1) and cloned into the *Bam*HI and the *Kpn*I sites of pApPh/*Avr*II to generate the transfer vector pApPh/*Avr*II-*egfp*-*Avr*II with *egfp* flanked by *Avr*II sites (Fig. 1A).

A gene encoding human mature thrombopoietin (hTPO) (GenBank, L36052.1, protein ID: AAC37566.1) was synthesized by SBS Genetech Co., Ltd. (Beijing, China) and cloned into the *Bam*HI and the *Eco*RI sites of the transfer vector pApM748BE (Wang et al. 2010) to create the plasmid pApM748BE/*htpo*, in which *htpo* was under the control of the polyhedrin promoter.
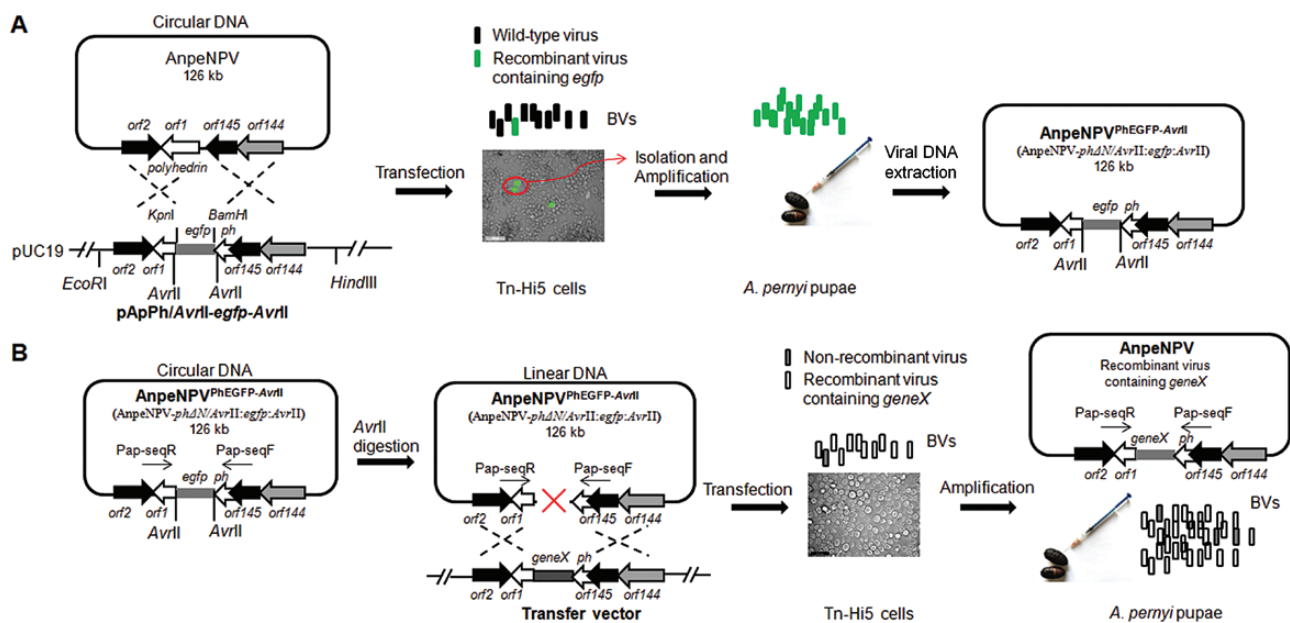
Generation and Isolation of AnpeNPV Derivatives

Approximately 1×10^6 Tn-Hi5 cells per well were seeded into a 6-well tissue culture plate (Falcon) 1–2 h prior to transfection. To generate a linearized derivative of AnpeNPV, 1 µg of pApPh/*Avr*II-*egfp*-*Avr*II plasmid DNA and 0.5 µg of wild-type AnpeNPV DNA were co-transfected into Tn-Hi5 cells with Cellfectin II (Cat No. 10362100; Gibco) according to the manufacturer's instructions. The EGFP-expressing cells were detected under UV light using an inverted microscope (DMI3000B; Leica, Wetzlar, Germany). The cell culture supernatant was harvested 5–6 d post-transfection and 100 µl was injected into *A. pernyi* pupae to amplify the recombinant virus. The end-point dilution method was used to isolate EGFP-expressing cells infected with the recombinant virus. After 10 d of incubation at 22–24°C, hemolymph was collected from infected pupae, diluted 1:100 with SF-900TM II medium, and filtered with a 0.45-µm filter (Lot No. 71621103; Sartorius, Göttingen, Germany). The filtered hemolymph was further diluted to 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} with SF-900TM II medium. Tn-Hi5 cells at a confluence of ~50% in each well of a 6-well tissue culture plate were infected with 1 ml of each diluted sample at 27°C for 1 h. The inoculums were removed after 1 h of infection. The cells were rinsed with 1 ml of SF-900TM II medium and then covered with 1 ml of 2% low-gelling-temperature agarose sterilized by autoclaving. Then, 1–2 ml of TNM-FH medium supplemented with 10% FBS was added to the solid overlay after the agarose set. The infected cells were incubated at 27°C for 5–6 d and the isolated cells expressing EGFP at the highest dilution were marked with a marker pen. The liquid overlay was discarded, and the solid overlay was rinsed with 1 ml of SF-900TM II medium. The marked cells containing the recombinant virus were picked up using sterile Pasteur pipettes and resuspended in 100 µl of SF-900TM II medium. Each viral supernatant was injected into a pupa of *A. pernyi*. After incubation at 22–24°C for 10 d, the hemocytes and other large tissue particles from the infected pupa were used to extract viral DNA using the Tissue gDNA Purification Kit (GD2211-02; Biomiga, San Diego, CA) to detect the purity of the recombinant virus by PCR with Pap-seqF and Pap-seqR primers

Table 1. Primers used for the construction of the baculovirus transfer vectors and qPCR in this study

Primer name	Primer sequences (5′–3′)	Purpose
PapHindIII-F	GG*AAGCTT*GCGCAAGAACGAGTCGTACTTG	PCR
PapBamHI-R	CC*GGATCC*TTATAGGAAATTTTACTACAAAG	PCR
PapKpnI-AvrII-F	AC*GGTACCTAGG*AAACTTATTGTCAACTGGAGCGG	PCR
PapEcoRI-R	CC*GAATTC*TAAGCACAAAAACGGCTCCC	PCR
Pegfp-BamHI	CC*GGATCC*ATGGTGAGCAAGGGCGAGGAGC	PCR
Pegfp-KpnI	CC*GGTACC*TTACTTGTACAGCTCGTCCATG	PCR
Pap-seqF	TTAACGCTTAGCCAGCAGCAG	PCR
Pap-seqR	TTCTTTACCGCTCCAGTTGAC	PCR
egfp	F: GTGAACCGCATCGAGCTGAA	qPCR
	R: GACGTTGTGGCTGTTGTAGTTG	
tpo	F: CTCTTGAATGGAACTCGTGG	qPCR
	R: CAGGGAGCCTGTGTCTGATG	
DNApol	F: CTCCGCTATAGAACACGTGCT	qPCR
	R: GTACATGAGCATGTCCGACC	

Relevant restriction sites incorporated in the primers are italicized.

Fig. 1. Schematic representation of the experimental design for generation, linearization and recombination of the AnpeNPV derivative carrying *Avr*II sites. (A) Recombination between the wild-type AnpeNPV DNA and pApPh/*Avr*II-*egfp*-*Avr*II plasmid DNA creates a recombinant virus containing *egfp* flanked by *Avr*II sites and under the control of the polyhedrin promoter, AnpeNPV^PhEGFP-*Avr*II. pApPh/*Avr*II-*egfp*-*Avr*II carries one fragment containing partial *orf144* (*lef-2*), full-length *orf145*, and the polyhedrin promoter (corresponding to nt 125168–126244*), *egfp*, and another fragment containing partial *orf1* (*polyhedrin*) and *orf2* (*orf1629*) (corresponding to nt 250–1268*). *The numbering system begins with the first nucleotide (A) of the initiation codon of *orf1* (*polyhedrin*) based on the sequence of AnpeNPV L2 (GenBank accession number: EF207986). (B) Treatment of the genomic DNA of AnpeNPV^PhEGFP-*Avr*II with *Avr*II endonuclease generates the linearized AnpeNPV DNA without *egfp*. The linear AnpeNPV DNA increases the recombination efficiency between the viral DNA and the transfer vector. Pap-seqF and Pap-seqR primers are used to verify the presence of the gene of interest (*geneX*) in the recombinant virus.

(Table 1). The PCR products were further cloned into the pMD18-T vector (TaKaRa) for sequencing to confirm the *Avr*II sites. The resulting derivative of AnpeNPV was named AnpeNPV^PhEGFP-*Avr*II (Fig. 1A).

Linearization of AnpeNPV DNA

The budded virus (BV) genomes of AnpeNPV^PhEGFP-*Avr*II were amplified in *A. pernyi* pupae. Briefly, hemolymph from an AnpeNPV^PhEGFP-*Avr*II-infected pupa was diluted 1:100 with SF-900TM II medium and filtered with a 0.45-µm filter. Approximately 50 µl was then injected into *A. pernyi* pupae. After incubation for 10–12 d

at 22–24°C, hemolymph samples were collected from infected pupae and centrifuged at 800 × *g* for 10 min to remove hemocytes and other large tissue particles. Viral particles were purified, and viral DNAs were extracted as previously described (Fan et al. 2007). To prepare the linear AnpeNPV DNA, ~10 µg of AnpeNPV^PhEGFP-*Avr*II DNA was digested with 80 units of *Avr*II (*Bln*I) endonuclease (1022A; TaKaRa) at 37°C overnight in 100 µl of buffer (20 mM Tris–HCl pH 8.5, 100 mM KCl, 10 mM MgCl$_2$, 1 mM dithiothreitol). The digested viral DNA was analyzed by electrophoresis on a 0.6% agarose gel with 0.5× TAE buffer (0.02 mol/liter Tris-HAc, 0.0005 mol/liter EDTA) (Fig. 1B).

Assay of the Infectivity of Linearized AnpeNPV DNA

Viral DNA (10 μg) was digested with *Avr*II as described above. Mock-digested viral DNA was prepared by the same procedure, except *Avr*II was omitted. After digestion, the viral DNA was heated at 70°C for 15 min, precipitated with ethanol, and dissolved in sterile TE buffer (pH 8.0). Then, 0.5 μg of the linearized AnpeNPV DNA or the mock-digested viral DNA was transfected into Tn-Hi5 cells with Cellfectin II as described above. After 5–6 d post-transfection, the cell culture supernatant was collected and injected into *A. pernyi* pupae at 100 μl per individual. After incubation at 22–24°C for 8 d, hemolymph samples from the infected pupae were used to extract genomic DNA of BVs using the QIAamp DNA Blood Mini Kit (Cat. No. 51104; QIAGEN GmbH, Hilden, Germany) following the manufacturer's instructions. To analyze the infectivity of the linearized AnpeNPV DNA and the mock-digested viral DNA, DNA polymerase (*DNApol*) levels were used as an indicator of virus proliferation (Martínez-Solís et al. 2017). The copy number of *DNApol* from 1 μg of BV DNA (copies/μg) was calculated using cycle threshold (Ct) values by quantitative real-time polymerase chain reaction (qPCR) with *DNApol* primers (Table 1) and obtained by comparison against a standard curve.

Analysis of Recombination Between the Linearized AnpeNPV DNA and Transfer Vectors

Briefly, 0.5 μg of linearized AnpeNPV DNA (or mock-digested viral DNA) and 1 μg of pApM748BE/*htpo* were co-transfected into Tn-Hi5 cells with Cellfectin II. Amplification of the recombinant virus in *A. pernyi* pupae and extraction of BV DNA were performed as described above (Fig. 1B). Recombination between the parental viral DNA and the transfer vector DNA was detected by PCR using BV DNA from the infected pupae as templates and Pap-seqF/Pap-seqR primers (Table 1). The copy numbers of *htpo* and *egfp* from 1 μg of BV DNA were determined by qPCR using specific primers (Table 1). The relative abundance of *htpo* (or *egfp*) was calculated by $2^{-\Delta Ct}$, where ΔCt is obtained by subtracting the Ct value for *DNApol* from the Ct value for *htpo* (or *egfp*) from each sample. The relative abundance of *htpo* was used to evaluate the recombinant virus efficiency between AnpeNPV DNA and pApM748BE/*htpo*, while the relative abundance of *egfp* was used to evaluate the nonrecombinant virus with *egfp* in infected pupae.

## Quantitative Real-Time Polymerase Chain Reaction

qPCR was performed by SYBR Green-based detection using the ABI 7500 Real-time PCR System (Applied Biosystems, Foster City, CA).

Plasmid DNAs containing the PCR products amplified by specific primers targeting *DNApol*, *htpo*, or *egfp* in the pMD18-T vector were used as templates to construct the standard curves. The standard curve for each primer pair was generated based on the linear relationship between the mean threshold cycle (Ct) values and serial dilutions ($-\log_{10}^{concentration}$) using software provided with the ABI 7500 System. The correlation coefficients (R^2) for *DNApol*, *egfp*, and *htpo* were 0.998, 0.988, and 0.998, respectively. Each reaction mixture contained 2 μl of viral genomic DNA, 0.4 μM each of the forward and reverse primers for the target gene, 10 μl of 2× SYBR Premix Ex Taq II (Tli RNaseH Plus) PCR master mix (TaKaRa), 0.4 μl of ROX Reference Dye II (50×), and nuclease-free water in a total volume of 20 μl. The thermal cycling conditions were as follows: 30 s at 95°C, followed by 40 cycles of 5 s at 95°C and 34 s at 60°C. Three independent infected pupae were used for viral DNA preparation. Three technical replicates were performed for each viral DNA. All statistical analyses were performed by paired *t*-tests using the GraphPad Prism (GraphPad Software Inc., San Diego, CA). Values of $P < 0.05$ were considered significant.

Results

Identification of an AnpeNPV Derivative Containing *Avr*II Restriction Endonuclease Sites

By screening a set of commercially available endonucleases using the reference sequence for AnpeNPV L2 (GenBank accession number: EF207986), we did not detect an *Avr*II site in the genomic DNA sequence. To create AnpeNPV derivatives containing *Avr*II sites, we first generated a baculovirus transfer vector, pApPh/*Avr*II-*egfp*-*Avr*II (Fig. 1A). Following recombination between the transfer vector (pApPh/*Avr*II-*egfp*-*Avr*II) and AnpeNPV genomic DNA in Tn-Hi5 cells, an AnpeNPV derivative, referred to as AnpeNPV[PhEGFP-*Avr*II], was isolated in which the partial polyhedrin sequence was replaced by the *egfp* sequence flanked by *Avr*II sites (Fig. 1A). The *egfp* gene was expressed under the control of the polyhedrin promoter in AnpeNPV and observed in Tn-Hi5 cells after infection with AnpeNPV[PhEGFP-*Avr*II] (Fig. 2A). As expected, sequencing results showed that the two *Avr*II sites were located at the 5′ and 3′ ends of *egfp*, respectively (data not shown). Treatment of the genomic DNA extracted from AnpeNPV[PhEGFP-*Avr*II] BVs with *Avr*II converted the viral DNA into two fragments, 0.72 kb of *egfp* and ~126 kb of the linearized AnpeNPV DNA lacking a portion of the polyhedrin gene sequence (1–249 nt) (Fig. 2B).
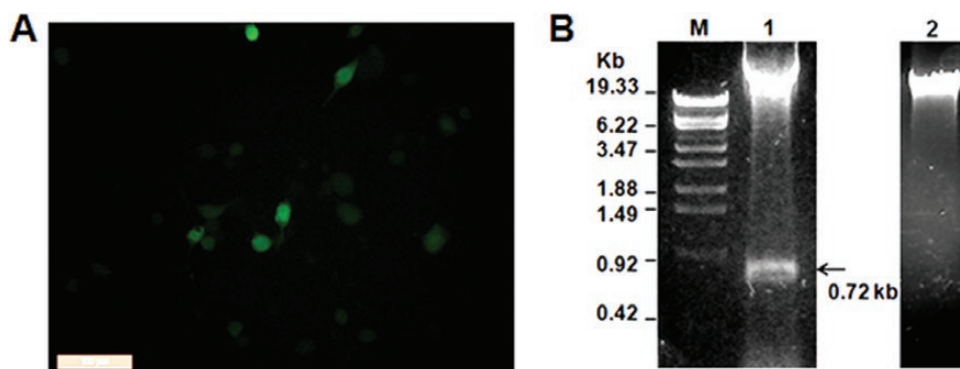
Fig. 2. Characterization of AnpeNPV[PhEGFP]-*Avr*II. (A) EGFP expression in AnpeNPV[PhEGFP]-*Avr*II-infected Tn-Hi5 cells was detected under UV light using an inverted microscope, 120 hpi. (B) Identification of AnpeNPV[PhEGFP]-*Avr*II DNA digested with *Avr*II endonuclease. M, λ/EcoRT14 I marker. 1, AnpeNPV[PhEGFP]-*Avr*II DNA digested with *Avr*II endonuclease. 2, undigested AnpeNPV[PhEGFP]-*Avr*II DNA.

Influence of Linearized AnpeNPV DNA on Virus Generation

In Tn-Hi5 cells transfected with the mock-digested viral DNA, many EGFP-expressing cells were observed (Fig. 3A). Relatively fewer cells expressing EGFP were observed in Tn-Hi5 cells transfected with the linearized AnpeNPV DNA; this was due to a small amount of undigested AnpeNPV$^{PhEGFP\text{-}AvrII}$ DNA mixed in the linearized AnpeNPV DNA (Fig. 3B).

Virus proliferation was further evaluated based on the copy number of *DNApol* of AnpeNPV in the hemolymph of infected pupae. This gene is essential for viral DNA replication (Rohrmann 2019). As determined by qPCR, the copy number of *DNApol* was 1.24×10^9/µg of BV DNA from pupae after mock-digested viral DNA transfection but was only 5.56×10^7/µg BV DNA from pupae infected with the linearized AnpeNPV DNA (Fig. 4A). The relative abundance of *egfp* in the BV DNA from pupae infected by transfection with linearized AnpeNPV DNA was less than 1/1,000 to that of the abundance after mock-digested viral DNA transfection (Fig. 4B). These results indicated that linearized AnpeNPV DNA substantially limited the generation of AnpeNPV in Tn-Hi5 cells, resulting in a reduction in viral progeny in *A. pernyi* pupae.

Influence of Linearized AnpeNPV DNA on Homologous Recombination

The linearized AnpeNPV DNA or mock-digested viral DNA was used as the parental viral DNA for cotransfection with the plasmid pApM748BE/*htpo* into Tn-Hi5 cells to generate the recombinant virus, which was further propagated in *A. pernyi* pupae. The *htpo* replaced *egfp* and was located at the polyhedrin locus in the recombinant virus. The results of PCR detection showed that of the two PCR products obtained from the BV DNA of *A. pernyi* pupae infected by cotransfection with the mock-digested viral DNA, one corresponded to *egfp* (approximately 0.8 kb) and the other corresponded to *htpo* (approximately 1.0 kb). A PCR product was obtained from the BV DNA of *A. pernyi* pupae infected by cotransfection with the linearized AnpeNPV DNA, corresponding to *htpo* (approximately 1.0 kb) (Fig. 5).

DNApol, *egfp*, and *htpo* were quantified by qPCR. Based on the *DNApol* copy number, progeny in infected pupae remained at similar levels after cotransfection with the linearized AnpeNPV DNA and the mock-digested viral DNA (Fig. 6A). The relative abundances of *egfp* and *htpo* showed opposite trends for the two cotransfections (Fig. 6B). The relative abundance of *egfp* was lower when linearized
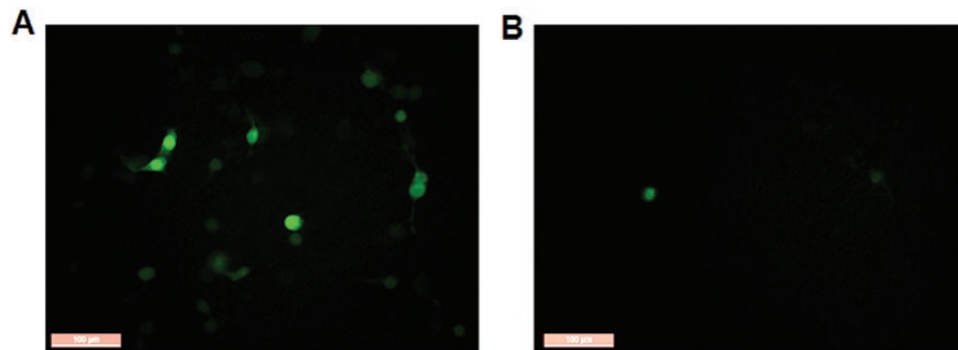

Fig. 3. Infectivity of linear and circular AnpeNPV DNA in Tn-Hi5 cells. (A) Tn-Hi5 cells transfected with the mock-digested viral (circular) AnpeNPV^{PhEGFP}-*Avr*II DNA, 120 hpi. (B) Tn-Hi5 cells transfected with the linearized AnpeNPV DNA, 120 hpi.
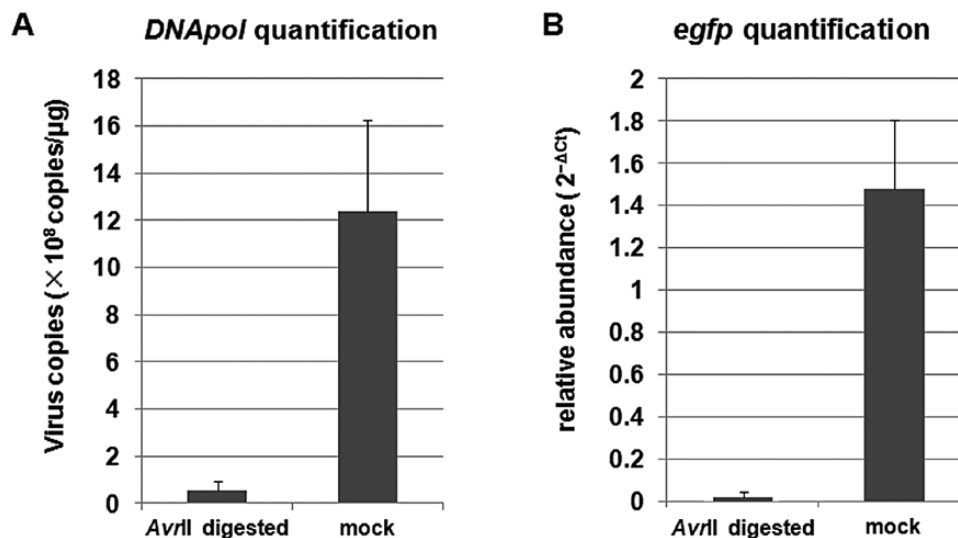

Fig. 4. qPCR analysis of viral DNA replication in infected *A. pernyi* pupae. (A) Total virus copies expressed as *DNApol* copies/µg of BV DNA. (B) Copies of parental virus with *egfp* are presented as the relative abundance of *egfp*. BV DNA was extracted from the hemolymph of the infected pupae. *Avr*II digested- linear AnpeNPV DNA. Mock- circular AnpeNPV^{PhEGFP}-*Avr*II DNA.

AnpeNPV DNA was cotransfected with the transfer vector, and the relative abundance of *htpo* was approximately 5.5-fold higher than that after cotransfection with the mock-digested viral DNA. These results suggested that the linearized AnpeNPV DNA dramatically reduced the generation of the parental virus and increased the recombination efficiency between the viral DNA and the transfer vector.

Discussion

A low frequency of homologous recombination has seriously hampered the acquisition of recombinants possessing modified sequences and the application of the AnpeNPV-based expression vector system has been developed as an efficient BEVS similar to the bacmid system. Linear baculovirus DNA is not able to replicate in insect cells but can increase the frequency of recombination with a cognate transfer vector (Kitts et al. 1990). In the present study, we successfully constructed an AnpeNPV derivative carrying the *egfp* gene flanked by *Avr*II restriction sites. The treatment of viral genomic DNA with *Avr*II produced linearized AnpeNPV DNA. qPCR-based quantification of *DNApol*, *egfp*, and *htpo* was used to evaluate the

infectivity of the linear viral DNA and recombination between the linear viral DNA and a transfer vector. Based on this analysis, linearized AnpeNPV DNA caused a marked reduction in parental BV production to approximately 4.5% of the parental BV production observed for circular AnpeNPV DNA and resulted in an approximately 5.5-fold higher relative abundance of the recombinant virus than that of the circular AnpeNPV DNA in *A. pernyi* pupae.

A previous study has shown that linear AcMNPV DNA is only 1/15th as infectious as circular viral DNA and increases the percentage of recombinant virus by up to 30% in *S. frugiperda* cells based on a plaque assay (Kitts et al. 1990). Unlike AcNPV infection in *S. frugiperda* cells, viral plaques are difficult to detect in AnpeNPV-infected Tn-Hi5 cells. *DNApol* is critical for viral DNA synthesis and replication and is stable during baculovirus infection and serial passages in permissive host cells (Vanarsdall et al. 2005, Martínez-Solís et al. 2017). In this study, a *DNApol*-based qPCR assay was used to indirectly monitor the replication of all virus types, including the parental virus, the recombinant virus, and the nonrecombinant virus. *egfp*-based and *htpo*-based qPCR assays were used to detect the parental virus and recombinant virus, respectively, in infected *A. pernyi* pupae. The qPCR results showed a clear difference in viral replication in pupae between the linearized and wild-type AnpeNPV DNA.

Different strategies are used for engineering linearized baculoviruses. Initially, a unique Bsu36I restriction site was introduced at the polyhedrin locus in AcMNPV, allowing linearization of the viral DNA. Although a 10-fold higher fraction of recombinants were obtained after cotransfection with the linear viral DNA than with the circular AcMNPV DNA, 60% of progeny viruses were nonrecombinants due to recircularization of the linear viral DNA (Kitts et al. 1990). The method was improved by the generation of an AcMNPV derivative in which three Bsu361 restriction sites were inserted into an essential gene (*orf1629*) downstream of the polyhedrin locus of AcMNPV. The linear AcMNPV DNA without *orf1629* can be produced by the digestion of the viral DNA with Bsu361. The function of *orf1629* is restored upon recombination between the defective AcMNPV DNA and an appropriate transfer vector, producing an infectious recombinant virus. This strategy led to a great reduction in nonrecombinant progeny viruses after cotransfection (Kitts and Possee 1993). However, the full-length ORF1629 is not essential for viral replication in both AcMNPV and BmNPV. A lack
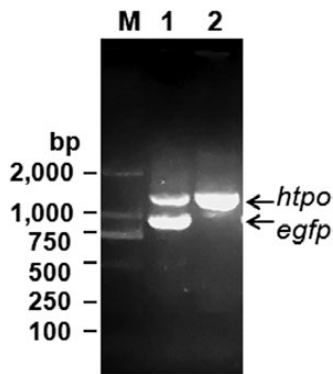
Fig. 5. PCR detection of recombinant virus in infected *A. pernyi* pupae. M, DL 2,000 DNA marker. 1, BV DNA extracted from *A. pernyi* pupae infected by cotransfection with the mock-digested viral (circular) AnpeNPV^PhEGFP_*Avr*II DNA. 2, BV DNA extracted from *A. pernyi* pupae infected by cotransfection with the linearized AnpeNPV DNA.
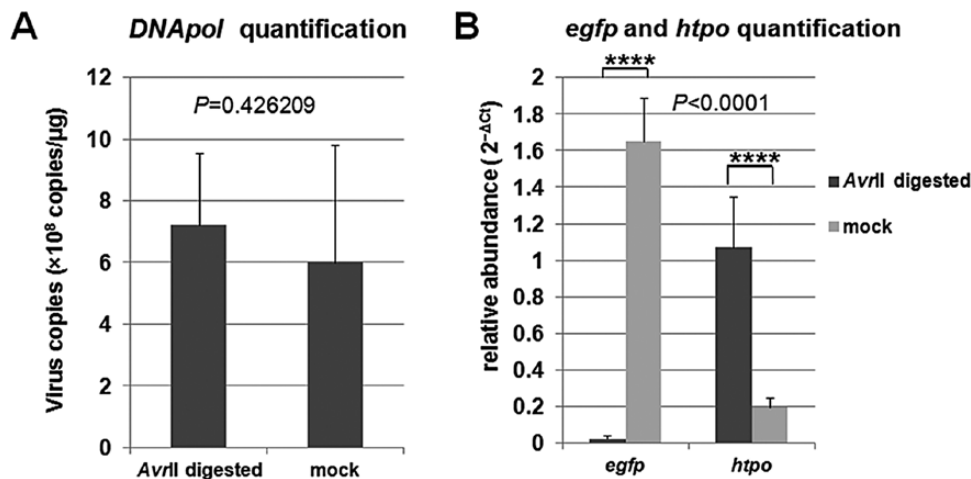
Fig. 6. qPCR analysis of the recombinant virus in infected *A. pernyi* pupae. (A) Total virus copies expressed as *DNApol* copies/µg of BV DNA. (B) Copies of parental virus with *egfp* are presented as the relative abundance of *egfp* (left). Copies of recombinant virus with *htpo* are presented as the relative abundance of *htpo* (right). BV DNA was extracted from the hemolymph of the infected pupae. *Avr*II digested- linear AnpeNPV DNA. Mock- circular AnpeNPV^PhEGFP-*Avr*II DNA.

of ORF1629 affects the formation of both BV and occlusion derived virus (ODV) and decreases the virus growth rate and yield (Gwak et al. 2019). *lef-2*, which is located upstream of the polyhedrin locus in baculoviruses, plays a dual role in viral DNA replication and very late transcription, including the transcription of *polyhedrin* and *p10* (Merrington et al. 1996, Sriram and Gopinathan 1998). The absence of *lef-2* abolishes BV production in cultured insect cells (Wu et al. 2010, Ono et al. 2012).

We initially constructed a transfer vector in which *egfp* was controlled by the polyhedrin promoter and flanked by fragments of *orf144* (*lef-2*) and *orf2* (*orf1629*). Two *Avr*II restriction sites were inserted upstream and downstream of the *orf144* (*lef-2*) ORF. We initially aimed to develop an AnpeNPV derivative with *Avr*II restriction sites at the *orf144* (*lef-2*) locus from cotransfection of the transfer vector and the wild-type AnpeNPV DNA. However, there were no *Avr*II restriction sites at the *orf144* (*lef-2*) locus in recombinant BVs after cotransfection (data not shown). This could be explained by DNA repair via recombination for the production of an infectious baculovirus in insects (Rohrmann 2019). As an alternative, we obtained an AnpeNPV derivative harboring *Avr*II restriction sites at the polyhedrin locus. This derivative can be used for the linearization of AnpeNPV DNA. Although the linearized AnpeNPV DNA could produce a small amount of nonrecombinant progeny due to end-joining reactions of the linear DNA, it undoubtedly improved the recombination rate to increase the proportion of recombinant viruses, thereby contributing to the efficient purification of recombinant viruses. Moreover, the linearized AnpeNPV DNA may be cloned directly using bacterial artificial chromosomes (BACs) to construct AnpeNPV bacmid system (Hilton et al. 2008, Abdallah et al. 2017). In summary, the newly developed AnpeNPV derivative provides a source of linear AnpeNPV DNA that will facilitate the isolation of recombinant viruses as well as the cloning of the AnpeNPV genome using BAC vectors for bacmid system construction.

Acknowledgments

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